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Author: Krzysztof Sitko, Szymon Rusinowski, Hazem M. Kalaji, Michał Szopiński, Eugeniusz Małkowski

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Photosynthetic Efficiency as Bioindicator of Environmental Pressure in *A. halleri*[OPEN]

Krzysztof Sitko,[a] Szymon Rusinowski,[b] Hazem M. Kalaji,[c,d] Michał Szopiński,[a] and Eugeniusz Małkowski[a,1]

[a]Department of Plant Physiology, Faculty of Biology and Environmental Protection, University of Silesia in Katowice, 40-032 Katowice, Poland
[b]Institute for Ecology of Industrial Areas, 40-844 Katowice, Poland
[c]SI Technology, 01-460 Warsaw, Poland
[d]Department of Plant Physiology, Warsaw University of Life Sciences SGGW, 02-776 Warsaw, Poland

ORCID IDs: 0000-0002-3833-4917 (H.M.K.); 0000-0003-0408-3600 (M.S.); 0000-0001-9804-8114 (E.M.).

In earlier ecophysiological studies that were conducted on *Arabidopsis halleri* plants, scientists focused on the mechanisms of Cd and Zn hyperaccumulation but did not take into consideration the environmental factors that can significantly affect the physiological responses of plants in situ. In this study, we investigated *A. halleri* that was growing on two nonmetalliferous and three metalliferous sites, which were characterized by different environmental conditions. We compared these populations in order to find differences within the metallicolous and nonmetallicolous groups that have not yet been investigated. The concentrations of several elements in the plant and soil samples also were investigated. To our knowledge, the concentration and fluorescence of chlorophyll were measured for *A. halleri* in situ for the first time. Our study confirmed the hyperaccumulation of Cd and Zn for each metallicolous population. For the metallicolous populations, the inhibition of parameters that describe the efficiency of the photosynthetic apparatus with increasing accumulations of heavy metals in the shoots also was observed. It was found that the nonmetallicolous plant populations from the summit of Ciemniak Mountain had larger antenna dimensions and chlorophyll content but a lower percentage of active reaction centers. To our knowledge, in this study, the internal high physiological diversity within the populations that inhabit metalliferous and nonmetalliferous sites is presented for the first time.

Plants have evolved anatomical and physiological mechanisms for the effective and selective uptake, transport, and accumulation of metals in their tissues (White, 2012). Some plant species have evolved strategies to cope with the toxic effects of the heavy metals (HM) that are present in excess in the environment as a result of human activity (e.g. mining and heavy industry, waste incineration, and sewage sludge application) and/or natural processes (e.g. volcanic emissions, weathering and erosion of soil, forest fires, and evaporation of the oceans; Baker et al., 2000; Hood, 2010; Kabata-Pendias, 2011). The accumulation and tolerance of HM in plants have developed in the course of their evolution as a response to abiotic stress. The hyperaccumulation and hypertolerance of HM, which are manifested as the accumulation of unusually high concentrations of metals in plant shoots without any visible symptoms of toxicity (Verbruggen et al., 2009, 2013; Krämer, 2010), are extreme examples of these traits. Approximately 500 taxa have been classified as HM hyperaccumulators (Brooks, 1998; Baker et al., 2000; Verbruggen et al., 2009; Krämer, 2010; van der Ent et al., 2013). Hyperaccumulators of As, Cd, Mn, Ni, Se, and Zn have been described and experimentally confirmed, whereas the hyperaccumulation of Ta, Co, Cr, and Cu is still in doubt (van der Ent et al., 2013) and the hyperaccumulation of Pb is questionable (Koubová et al., 2016). For the micronutrients that are classified as HM (e.g. Cu, Mn, and Zn), three concentration ranges were distinguished that determine the physiological responses of plants: deficiency, optimal concentrations, and toxic concentrations. Nonessential elements (e.g. Cd and Pb) are not necessary for plant growth, and there are no growth-limiting low concentrations for such elements (Lin and Aarts, 2012).

The specific groups of plants that occur in areas that have been contaminated with HM are eumetallophytes and pseudometallophytes, which are species that are found only in metalliferous sites and those that appear in both metalliferous and nonmetalliferous habitats, respectively (Pauwels et al., 2005, 2006). *Arabidopsis halleri* (*Cardaminopsis halleri*, Haller's rockcress) is a multimetallicolous hyperaccumulator (Cd and Zn), a pseudometallophyte, and a close relative species to

[1] Address correspondence to eugeniusz.malkowski@us.edu.pl.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Eugeniusz Małkowski (eugeniusz.malkowski@us.edu.pl).

K.S. and E.M. designed the experiments; K.S. performed most of the experiments; S.R. and M.S. provided technical assistance and statistical analysis; H.M.K. and E.M. supervised the writing; all of the authors contributed to the writing.

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Arabidopsis thaliana, which is a model plant. Very high levels of Cd and Zn accumulation in its shoots and its close genetic relatedness to *A. thaliana* made *A. halleri* a model species for detailed studies of the mechanisms of HM hypertolerance and hyperaccumulation (Pauwels et al., 2006; van der Ent et al., 2013; Verbruggen et al., 2013). Nonmetallicolous (NM) populations of this species, which are associated with uplands (200 m above mean sea level [AMSL]) and mountain habitats (2,200 m AMSL) in Europe, are located mainly in the Carpathian Mountains, the Alps, and the Bavarian Highlands (Fabiszewski, 1986, Clauss and Koch, 2006; Pauwels et al., 2006; Koch and Matschinger, 2007; Wasowicz et al., 2016). Pauwels et al. (2005), on the basis of chloroplast DNA haplotypes (chlorotypes) of selected European metallicolous (M) and NM populations, proposed that hyperaccumulation evolved independently in different regions and that M populations are more closely related to the nearest NM mountain populations than to any M population from another part of Europe. In addition, eight suprahaplotypes of the plants were distinguished on the basis of chloroplast DNA, and among them, the oldest is in the western Carpathians (Tatra Mountains; Koch and Matschinger, 2007). The allelic richness of populations from the western Carpathians compared with that in Upper Silesia was confirmed by Wasowicz et al. (2016), which suggests that the populations from the Tatra Mountains may have been the origin of the populations that are located in Upper Silesia. Furthermore, Stein et al. (2017) recently showed that there is a high degree of variation in the accumulation of different elements, including Cd and Zn, in the populations of *A. halleri* from different locations. Hence, the data presented above suggest that the origin of the NM and M populations may have an impact on the results of comparative research on the hyperaccumulation of HM or other physiological processes in *A. halleri*. Therefore, it seems to be necessary to conduct investigations on populations that are as closely related as possible. Therefore, a comparison of the populations from the Tatra Mountains and Upper Silesia, which are closely related, would be valuable.

Currently, the mechanisms of the uptake, transport, and hyperaccumulation of Zn by *A. halleri* are well understood at both the whole-plant (Verbruggen et al., 2009) and cellular (Cosio et al., 2004; Marquès et al., 2004) levels, including the uptake and storage mechanisms (Aucour et al., 2011). The roles of transporters (Nouet et al., 2015) and chelating agents, such as nicotianamine, also were investigated recently (Deinlein et al., 2012; Tsednee et al., 2014). To some extent, the role of rhizosphere microorganisms in hyperaccumulation also has been identified (Farinati et al., 2009). The mechanism of Cd hyperaccumulation, however, remains the subject of intensive studies. The role of the Ca transporter CAX1 in Cd tolerance has been postulated (Baliardini et al., 2015), and the different mechanisms of detoxification (Isaure et al., 2015; Meyer et al., 2015) as well as the effect of Cd on the pool of free amino acids (Zemanová et al., 2013) were examined. The sites of accumulation in plant tissues also have been investigated. In leaves of *A. halleri*, the highest concentrations of Cd and Zn were found in mesophyll cells (Küpper et al., 2000), and leaf edges were the most Cd-enriched tissues (Huguet et al., 2012). In contrast, different patterns of accumulation of both metals were observed in *Noccaea caerulescens*, the second well-known hyperaccumulator of Cd and Zn (Krämer, 2010; Mandáková et al., 2015). It was proved that, in this species, the concentration of Zn was the highest in vacuoles of epidermal cells (Frey et al., 2000), whereas the highest concentration of Cd was observed in the path of water migration from the vascular cylinder to epidermal cells, which is in line with passive Cd transport by the transpiration stream (Wójcik et al., 2005).

Other physiological processes, such as photosynthesis, have rarely been examined in *A. halleri* to date (Cho et al., 2003; Meyer et al., 2010). On the other hand, a proteomics analysis of *A. halleri* shoots showed that photosynthesis-related proteins are up-regulated in plants that have been exposed to Cd or Zn, suggesting that both metals exert an influence on this physiological process (Farinati et al., 2009). Moreover, *A. halleri* accumulates Cd and Zn predominantly in mesophyll cells, in which photosynthesis occurs. Hence, investigations of the photosynthetic activity of *A. halleri* seem to be particularly useful to assess the plant physiological status and could provide new data on the mechanism of the adaptation of both NM and M populations to their habitats.

Measuring chlorophyll *a* fluorescence is a common method for monitoring the photosynthetic activity of plants and algae, which allows photosynthesis under stress to be studied in situ and in the laboratory, thus providing rapid information on the electron transfer reactions involving PSII and/or PSI in ecotoxicological research (Baker, 2008; Kalaji et al., 2011, 2012, 2014b). Plants, algae, and cyanobacteria emit fluorescence at a wavelength of 660 to 780 nm, the source of which is PSII. In the analysis of the chlorophyll *a* fluorescence transients, it is generally accepted that the state of the PSII reaction center (RC) is defined only by the redox state of the PSII primary quinone acceptor (Q_A). When Q_A in an RC is reduced (Q_A^-), the RC is closed and the chlorophyll *a* fluorescence is high, whereas when Q_A is in the oxidized state, the RC is open and the fluorescence of the antenna is quenched (Strasser et al., 2004). HM cause a negative impact on the light phase of photosynthesis and the efficiency of the photosystem. For example, Cd and Zn have direct effects on the activity of Light-Harvesting Protein II, PSII, the fraction of Q_A, and the oxygen-evolving complex (OEC; Myśliwa-Kurdziel et al., 2002; Kalaji and Loboda, 2007; Tuba et al., 2010).

The accumulation of HM in tissues of hyperaccumulators (e.g. *A. halleri* and *N. caerulescens*) was the major goal of the studies conducted on these species in situ (Bert et al., 2000, 2002; Dahmani-Muller et al.,

2000; Assunção et al., 2003; Pauwels et al., 2006; Stein et al., 2017). On the other hand, there are no reports describing other physiological processes (e.g. photosynthesis) in plants under environmental pressure. In consequence, there is no information available concerning the physiological status of *A. halleri* plants growing in their natural environment. This seems particularly important because *A. halleri* inhabits various habitats, from upland forests to alpine meadows, which differ in the quality and quantity of irradiance, annual precipitation, and temperature as well as in the concentration of HM in the soil. Taking into account that *A. halleri* is able to grow in such different habitats, it could be hypothesized that different populations should be diverse in their physiological status, regardless of whether the population is NM or M. Therefore, in this study, we focused our attention on investigating the physiological diversity within the NM as well as the M populations of *A. halleri* in situ, which have not yet been examined in detail. For this reason, three M and two NM populations of *A. halleri* from different habitats, which are closely related genetically, were chosen and photosynthesis under the influence of the environmental conditions (ecological pressure) was investigated. The concentrations of the elements in the roots and shoots also were examined. The principal component analysis (PCA) method was used to describe the relationship between the accumulation of Cd, Pb, and Zn in the shoots, the environmental conditions, and the efficiency of the photosynthesis of plants in situ.

RESULTS

Accumulation of Elements

Population P accumulated the highest concentrations of Cd and Zn in the shoots (1,003 and 24,320 $\mu g\ g^{-1}$, respectively; Table III), which corresponds with the highest content of both metals in the soil (Table II). It is noteworthy that the *A. halleri* at this site accumulated the lowest iron concentration compared with the other M populations (only 109.4 $\mu g\ g^{-1}$). The remaining M populations (B and W) also showed hyperaccumulations of Cd and Zn, but the concentrations in the shoots did not exceed 210 and 12,500 $\mu g\ g^{-1}$, respectively (Table III). Both were also characterized by the highest iron content in the shoots compared with the other sites. The shoot:root ratio of Zn for the M populations B, P, and W were as follows: 1.88, 2.54, and 1.16. For Cd, this ratio was 2.26, 1.95, and 1.98, respectively. (Population and site names are defined in Fig. 1 legend.)

Hyperaccumulation was not observed in the NM populations, since the concentrations of HM in the shoots were always much lower than in the roots (Table III). Population R accumulated significantly higher concentrations of Ca and Mn in the shoots compared with the other examined populations. It is noteworthy that, in the C population, the concentration of Mg in the shoots (5,686.6 $\mu g\ g^{-1}$) was highest compared with all of the other sites that were studied.

Characteristics of the Photosynthetic Apparatus

The definitions of parameters that are used to describe photosynthesis are presented in Supplemental Table S2. An increase in the fluorescence of chlorophyll *a* was shown as a typical OJIP transient, since fluorescence was measured at the time of 1 s on a logarithmic scale (Fig. 1A; Strasser et al., 2004). Between O (the level of fluorescence when plastoquinone [Q_A] is maximally oxidized, which means that the PSII RCs are open) and P (Q_A is maximally reduced and RCs are closed), there are two distinct steps/bands, J (0.002 s) and I (0.02 s). Phase O-J is highly light dependent and carries information about the size of the antennas and the communication between the RCs of PSII. The I-P phase, which is also called the thermal phase, is associated with a reduction in the residual electron transport chain (e.g. inactivation of ferredoxin-$NADP^+$ reductase [FNR]; Schansker et al., 2006). In addition, changes in the kinetics of the OJIP fluorescence increase were revealed by calculating the differences in the variable fluorescence curves [ΔV_t, where $V_t = (F_t - F_0)/(F_m - F_0)$, V_t represents variable fluorescence, F_0 represents minimal fluorescence, and F_m represents maximal fluorescence; Fig. 1B]. ΔV_t was calculated according to Kalaji et al. (2014a) as the difference between the mean values

Table I. *Geographical and climatic characteristics of the investigated populations*

Parameters	Name of the Site				
	P	B	W	C	R
Edaphic type	M	M	M	NM	NM
Location	Piekary Śląskie	Bukowno	Wełnowiec	Ciemniak Mountain	Kościeliska Valley
Altitude (m AMSL)	256	339	302	2,095	1,000
Temperature (°C)[a]	8.3	7.9	8.6	−0.5	4.8
Precipitation (mm)[a]	670	710	890	1,715	1,080
Latitude	50°22′00.6′′N	50°16′58.1′′N	50°17′13.0′′N	49°13′51.8′′N	49°15′18.1′′N
Longitude	18°58′18.4′′E	19°28′43.4′′E	19°01′32.0′′E	19°54′12.6′′E	19°52′03.2′′E
Habitat	Meager grassland	Woody area	Meager grassland	Alpine meadow	Rock niches
Aspect	–	–	–	SW	N

[a]The average annual temperature and precipitation values for the period 1982 to 2012 (climate-data.org).

Table II. *Physicochemical soil properties*

Presented data are means ± SE ($n = 8$, except for C, where $n = 6$). Means followed by the same letter in a row are not significantly different from each other using the LSD test ($P \leq 0.05$). EC, Electrical conductivity.

Parameters	Name of the Site				
	P	B	W	C	R
Physical properties of the soil					
pH (H_2O)	6.30 ± 0.06 c	6.76 ± 0.01 b	6.30 ± 0.08 c	6.63 ± 0.05 b,c	6.93 ± 0.06 a
pH (KCl)	6.26 ± 0.05 a,b	6.14 ± 0.04 b	5.75 ± 0.07 c	6.41 ± 0.06 a	6.18 ± 0.05 b
EC ($\mu S\ cm^{-1}$)	108.5 ± 11.5 d	145.3 ± 5.1 c,d	163.0 ± 14.7 b,c	545.0 ± 21.3 a	418.1 ± 18.3 a
Total concentration of the elements in the soil ($\mu g\ g^{-1}$)					
Ca	11,210 ± 2,255 b	4,136 ± 336 d	6,638 ± 644 c,d	9,192 ± 1,165 c	33,432 ± 533 a
Cd	665.3 ± 51.9 a	28.6 ± 2.2 b	80.7 ± 7.3 b	4.6 ± 0.1 b	6.1 ± 0.1 b
Cu	125.6 ± 13.6 b	23.6 ± 1.7 d	427.8 ± 33.4 a	24.9 ± 0.4 d	39.3 ± 0.4 c,d
Fe	17,830 ± 1,070 b	13,090 ± 262 c	42,810 ± 2,218 a	20,310 ± 869 b	18,462 ± 170 b
Mg	6,809 ± 1,137 a	5,569 ± 157 b	3,036 ± 200 b	3,425 ± 615 b	8,534 ± 106 a
Mn	1,047 ± 103 a	1,084 ± 18 a	1,082 ± 51 a	779.1 ± 21.7 b	711.3 ± 10.2 b
Pb	22,450 ± 3,599 a	1,053 ± 91 b	3,856 ± 260 b	208.3 ± 1.4 b	234.0 ± 2.1 b
Zn	24,580 ± 2,329 a	7,168 ± 274 c	15,710 ± 1,334 b	428.6 ± 2.7 d	921.1 ± 9.4 d
Concentration of the phytoavailable forms of HM in the soil ($\mu g\ g^{-1}$)					
Cd	60.8 ± 2.1 a	0.82 ± 0.09 b	2.77 ± 0.20 b	0.02 ± 0.00 c	0.03 ± 0.00 c
Pb	12.6 ± 2.4 a	0.29 ± 0.03 b	1.27 ± 0.04 b	0.18 ± 0.02 b	0.14 ± 0.01 b
Zn	966.1 ± 16.3 a	324.5 ± 35.4 b	950.3 ± 33.5 a	0.85 ± 0.11 c	0.38 ± 0.03 d

of the fluorescence for the population and the mean values of the fluorescence of the plants from the R site, which was used as a reference population because it had the best PSII performance, including the highest value to describe the active RC per cross section (CS) of an examined leaf (RC/CS), the lowest energy dissipation per CS (DI/CS), and the highest maximum quantum efficiency of PSII (φP_0). The greatest changes in fluorescence were observed for plants from M habitats, and they were visible in steps J (V_J) and I (V_I; Fig. 1A).

For ΔV_t, there were at least three peaks: ΔK (0.0003 s), ΔJ (0.002 s), and ΔI (0.02 s). All of these steps are associated with changes in the activity of the protein complexes. ΔK provides information about the degree of the disengagement of the OEC, ΔJ is associated with the accumulation of Q_A^-, and ΔI is associated with the inactivation of the FNR (Kalaji et al., 2014a). Figure 1B clearly shows that the M populations are characterized by the presence of all three peaks but that the ΔV_t characteristic is different depending on the examined populations. Populations P and W were characterized by high values at ΔK, whereas population B showed the highest value at ΔI. The ΔV_t curves of the NM plant C population did not deviate significantly from the values of the reference population R. Population C had identical values at ΔJ, although higher peaks were observed at ΔK and ΔI (Fig. 1B).

The R population was characterized by the highest value of φP_0 (Table IV). φP_0 also reflects the general physiological status of a plant and is equal to the value of the F_v/F_m parameter. The plants from population B were characterized by φP_0 that was similar to plants from population R, whereas in the plants from the C and P sites, φP_0 was lowest. The same relationship was observed for the quantum efficiency of electron transport (φE_0). In the case of the parameters that directly describe the OJIP transient, the most important are F_0 (minimal fluorescence), F_m (maximal fluorescence), and F_v ($F_v = F_m - F_0$). The highest values of F_v were measured for the NM habitats and population B and the lowest for M population P. Moreover, the NM plants were characterized by the highest values of the area parameter (reflecting pools of plastoquinone). The NM plants also were characterized by higher values of the ΨE_0 and φE_0 parameters, which describe the yield of the electron transport and the longest time to reach the maximum fluorescence, respectively (Table IV). The highest values of the quantum yield of energy dissipation (φD_0) were measured for the plants that inhabit Piekary Śląskie and Ciemniak Mountain. The highest values of the parameters that describe the probability (δR_0) and yield (φR_0) of a reduction in the end electron acceptors at the PSI acceptor site were observed for the plants from the NM sites and for population P (Table IV).

Plants from the summit of Ciemniak Mountain were characterized by the highest chlorophyll content in the leaves (13.07 relative units) compared with the other populations (Table IV). By contrast, population B had the lowest chlorophyll content (4.80 relative units).

Phenomenological Energy Flux

The phenomenological pipeline models of energy fluxes through the leaf CSs of the plants representing each site are presented in Figure 2. The R population was characterized by the highest yield of PSII compared with the other sites. At relatively low values of energy absorption by the CS of the leaves (ABS/CS) that were observed, they had high values of energy trapping (TR/CS) and electron transport (ET/CS) and the

Table III. *Accumulation of the elements in the plants*

Presented data are means ± SE ($n = 8$, except for C, where $n = 6$). Means followed by the same letter in a row are not significantly different from each other using the LSD test ($P \leq 0.05$).

Parameters	Name of the Site				
	P	B	W	C	R
Edaphic type	M	M	M	NM	NM
Accumulation of elements in shoots ($\mu g\ g^{-1}$)					
Ca	11,700 ± 259 c	16,920 ± 984 b	16,420 ± 799 b	18,170 ± 366 b	30,430 ± 1,356 a
Cd	1,003 ± 28 a	203.2 ± 25.0 b	129.0 ± 10.1 c	7.00 ± 0.6 d	5.8 ± 0.2 d
Cu	10.0 ± 0.4 b	10.3 ± 0.5 b	16.1 ± 0.7 a	16.1 ± 0.6 a	14.9 ± 0.3 a
Fe	109.4 ± 14.6 c	356.8 ± 17.4 a	352.0 ± 52.2 a	117.6 ± 7.9 b,c	205.9 ± 22.1 b
Mg	4,895 ± 217 b,c	4,947 ± 289 b	4,643 ± 257 c	5,686 ± 82 a	3,770 ± 248 d
Mn	34.4 ± 2.2 c	57.5 ± 3.0 a	47.7 ± 2.8 b	30.4 ± 1.5 c	41.8 ± 1.3 b
Pb	401.3 ± 34.8 a	25.6 ± 2.5 b	58.9 ± 1.7 b	33.1 ± 0.7 b	30.2 ± 0.3 b
Zn	24,320 ± 1,595 a	11,870 ± 209 b	12,210 ± 966 b	3,529 ± 480 c	2,170 ± 91 c
Accumulation of the elements in roots ($\mu g\ g^{-1}$)					
Ca	1,675 ± 28 c	3,642 ± 115 b	2,075 ± 216 c	1,772 ± 171 c	5,326 ± 160 a
Cd	514.7 ± 45.1 a	90.4 ± 8.7 b	64.6 ± 7.9 b	45.3 ± 1.2 b	14.7 ± 1.0 b
Cu	21.6 ± 1.4 c	28.3 ± 3.4 b	37.5 ± 5.3 b	110.0 ± 3.0 a	33.9 ± 3.1 b
Fe	157.9 ± 20.3 b	300.6 ± 27.0 a	142.2 ± 12.6 b	188.1 ± 8.5 b	131.1 ± 9.4 b
Mg	1,455 ± 99 c	2,515 ± 101 a	2,161 ± 216 b	2,548 ± 111 a	2,407 ± 28 a,b
Mn	12.9 ± 0.8 c	55.3 ± 2.4 a	25.5 ± 2.9 b	9.7 ± 0.4 c	11.5 ± 1.0 c
Pb	3,302 ± 156 a	107.8 ± 12.1 b	335.8 ± 35.0 b	259.3 ± 9.3 b	97.8 ± 5.8 b
Zn	9,580 ± 472 b	6,503 ± 494 c	10,500 ± 1,120 b	24,590 ± 1,438 a	5,678 ± 736 c

highest percentage of active reaction centers (RC/CS) simultaneously with the low energy dissipation (DI/CS). The highest absorbance of light was observed for the plants from the C site. Furthermore, these plants were characterized by the highest values of TR/CS, ET/CS, and DI/CS simultaneously. On the other hand, the percentage of active RCs was only 68% of that of the R population and corresponded with the state of the RC/CS of population W. The highest efficiency of PSII of the M population was measured for population B, whereas the lowest was measured for population P, which inhabits the meadow in Piekary Śląskie. In the P population, the value of RC/CS only reached 60% of that observed in the reference population (R). Plants in population P also were characterized by the lowest values of ABS/CS, TR/CS, and ET/CS.

Influence of the Environment on the Physiological Parameters and the Accumulation of Metals

PCA showed many relationships between the environmental and physiological factors of the *A. halleri* populations that were studied (Fig. 3). The PCA factors explained 89.81% of the variation in the data set. The first axis (ordinates) explained 68.19% of the variation and clearly separated the NM and M populations. PCA confirmed that the NM populations were characterized by a greater plastoquinone pool (area parameter) and φE_0 compared with plants from the metalliferous sites, which indicates a better performance of PSII. The second axis, which explained 21.62% of the variation in the data, separated the groups of parameters that were related to climate conditions and chlorophyll content. Based on the analysis, there was a negative correlation between all of the photosynthetic parameters and HM accumulation in the shoots and the concentration in the soil (Supplemental Table S1). A positive relation between precipitation, temperature, and the PSII parameters also was found. It should be emphasized that a negative linear relationship between φE_0 and Cd accumulation in the aerial parts of plants was observed (Fig. 3).

It is noteworthy that φP_0 correlated with both the environmental and physiological factors (Supplemental Table S1). These results confirm that φP_0 can be used to predict the general physiological status of a plant. Based on this parameter, the R population had the best and population P had the worst physiological status (Fig. 3).

DISCUSSION

Plant Mineral Composition

Stein et al. (2017) found that the hyperaccumulation of Cd is not observed in most of the NM populations that grow on soils with a natural concentration of both metals. Two NM populations (C and R) that were investigated in this study accumulated low amounts of Cd in their shoots (Table II); thus, hyperaccumulation also was not detected in these plants. However, Stein et al. (2017) revealed that the population located in the vicinity of the R population (Łysa Polana, 955 m AMSL) was characterized by a high diversity of Cd accumulation in leaves (ranging from 32 to 302 mg kg^{-1} dry weight). On the other hand, in this study, all of the individual plants from the M populations accumulated Cd in the shoots above 100 mg kg^{-1} dry weight. By contrast, Stein et al. (2017) showed that a large number

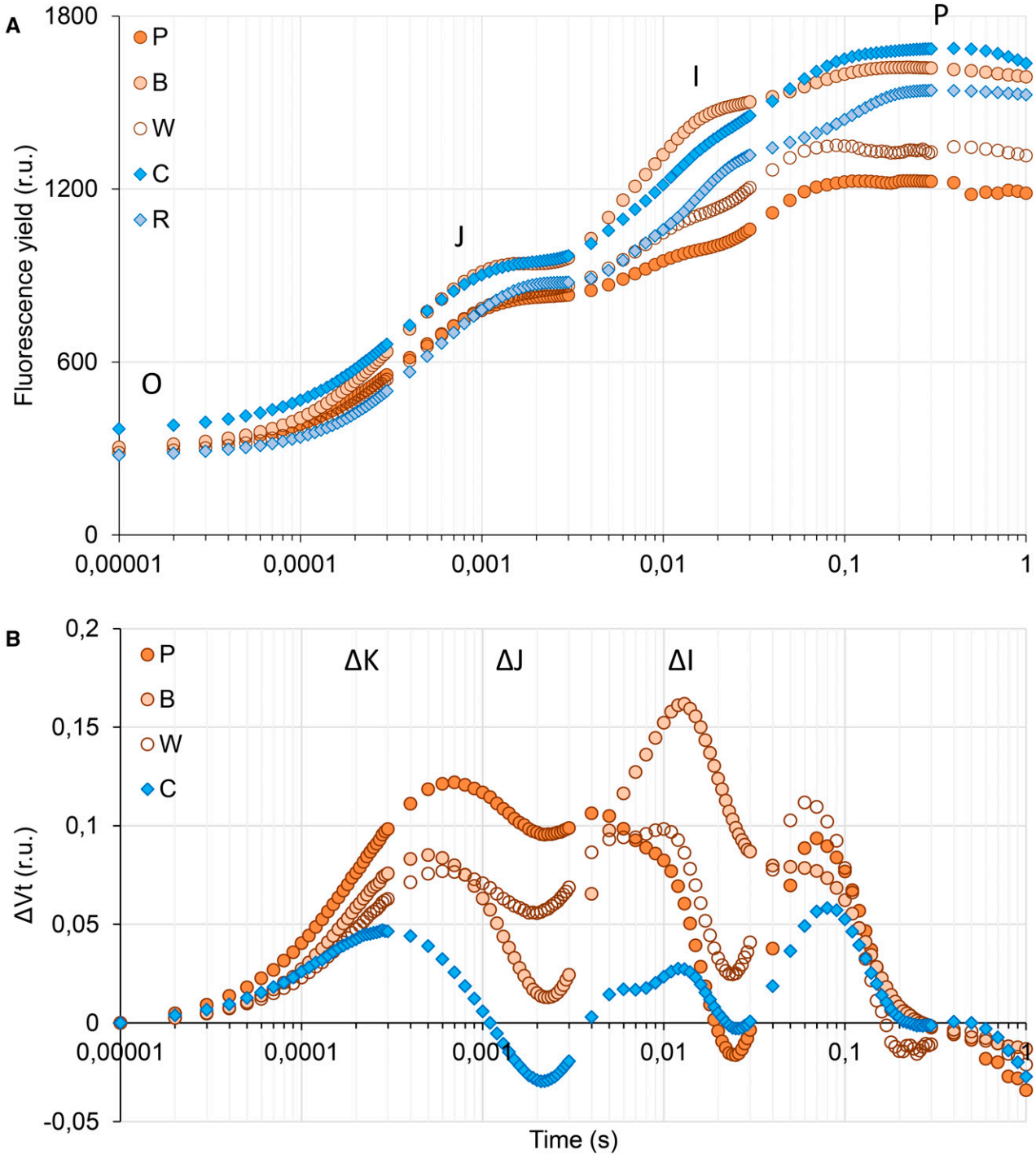

Figure 1. Chlorophyll *a* fluorescence induction curves of the *A. halleri* populations (A) and the effects of environmental pressure on the relative variable fluorescence [$\Delta V_t = ((F_t - F_0)/F_v) - V_{tF}$] of the populations that were studied (B). M populations are indicated as orange circles, and NM populations are presented as blue squares. For ΔV_t analysis, the fluorescence of population R was the reference and equaled 0. Abbreviations of the site names are as follows: P, Piekary Śląskie; B, Bukowno; W, Wełnowiec; C, Ciemniak Mountain; R, rocky site in the Kościeliska Valley. Values are means (n = 60). r.u., Relative units.

of individual plants from different M populations (including populations B and W) had a Cd concentration in the leaves below 100 mg kg^{-1} dry weight. For example, this was demonstrated in the W population from Katowice-Wełnowiec, in which five of 12 specimens had concentrations of Cd lower than 80 mg kg^{-1} dry weight (Stein et al., 2017). Differences in Cd concentrations in shoots between the results presented in

this study and those obtained by Stein et al. (2017) may be caused by the time of sampling (October for Stein et al. [2017] and August for the data presented here) or the weather conditions in the year of sampling.

There are many nonspecific as well as specific interactions among mineral nutrients in plants. Specific interactions (e.g. competition between nutrients at the cellular level or replacement of one nutrient by another; White, 2012) also are important in evaluating the critical toxicity concentration. The results presented for plants from populations B and W suggest an increase of the Fe accumulation in the shoots with an increasing accumulation of Zn and Cd in the shoots (Table II). A possible mechanism of the interaction between Zn and Fe, which prevents the toxic effect of Zn, was proposed by Shanmugam et al. (2013). Recent reports also confirmed an increase in the accumulation of iron in the leaves of plants characterized by a hyperaccumulation of Zn and Cd (Stein et al., 2017).

It has been shown that Cd enters plants through the same transport routes as Zn (Zhao et al., 2006). The role of the Ca antiporter CAX1 in Cd tolerance also has been postulated (Baliardini et al., 2015). Therefore, competition between Cd^{2+}, Zn^{2+}, and Ca^{2+} ions should reflect changes in the accumulation of these elements. For all of the M populations, the Ca concentrations showed a significant decrease compared with the reference population R (Table II). Results reported by Meyer et al. (2015) and Stein et al. (2017) showed a stimulating effect of Cd on the accumulation of Mg in the shoots of *A. halleri*. Our research also confirmed a higher accumulation of Mg in the shoots of the M populations compared with the R population. Although the NM population C was characterized by the highest concentration of Mg, this phenomenon will be discussed further in the section on the photosynthetic apparatus. Unfortunately, there is a dearth of articles discussing the effect of HM on the mineral composition of plants growing in situ.

Stein et al. (2017) investigated sites B and W, which are presented in this study, and some populations from the Tatra Mountains region, from which it could be possible to select a population similar to the R population. It was found that the relationships between element concentrations (Ca, Cd, Cu, Fe, Mg, Mn, Pb, and Zn) analyzed in plants were qualitatively and quantitatively similar in both studies.

The Impact of Environmental Factors on the Photosynthetic Apparatus

The toxic effect of HM on photosynthesis has been investigated at the cell, tissue, organ, and whole-plant levels (Mallick and Mohn, 2003; Maksymiec et al., 2007; Baker, 2008). The results for the M populations presented here describe the toxic effect of HM on the PSII activity parameters, such as φP_0, that are measured in situ (Table IV). There is a dearth of articles presenting the results of PSII activity in situ, but some studies that have been performed in controlled conditions showed that this parameter (also known as F_v/F_m) decreases under the toxic effects of Cd (Cho et al., 2003; Zhou and Qiu, 2005; Küpper et al., 2007; Maksymiec et al., 2007), Pb (Kalaji and Loboda, 2007), and Zn (Cho et al., 2003; Przedpelska-Wasowicz and Wasowicz, 2013). The hyperaccumulators (e.g. *A. halleri*, *Thlaspi caerulescens*,

Table IV. *Characteristics of the photosynthetic apparatus*

Presented data are means ± SE ($n = 60$). Means followed by the same letter in a row are not significantly different from each other using the LSD test ($P \leq 0.05$). Parameters are as follows: F_0, minimal fluorescence, when all of the PSII RCs are open (at time 0); F_m, maximal fluorescence, when all of the PSII RCs are closed; F_v, maximum variable fluorescence; φP_0, maximum quantum yield of the primary PSII photochemistry; ΨE_0, probability (at time 0) that a trapped exciton moves an electron into the electron transport chain beyond Q_A^-; φE_0, quantum yield for electron transport from Q_A^- to plastoquinone; δR_0, probability that an electron from the intersystem electron carriers will move to reduce the end acceptors at the PSI acceptor side; φR_0, quantum yield for the reduction of the end electron acceptors at the PSI acceptor side; φD_0, quantum yield (at time 0) of energy dissipation; T_{Fm}, time (in ms) to reach F_m; Area, total complementary area between the fluorescence induction curve and $F = F_m$ (total plastoquinone pool).

Parameters	Name of the Site				
	P	B	W	C	R
Edaphic type	M	M	M	NM	NM
PSII characteristics (relative units)					
F_0	281 ± 11 b	279 ± 6 b	277 ± 4 b	371 ± 27 a	267 ± 5 b
F_m	1,268 ± 30 d	1,614 ± 38 a,b	1,310 ± 30 c	1,705 ± 43 a	1,575 ± 32 b
F_v	987 ± 34 c	1,335 ± 32 a	1,033 ± 26 b	1,334 ± 57 a	1,307 ± 27 a
φP_0	0.77 ± 0.01 b	0.83 ± 0.00 a	0.81 ± 0.00 a	0.78 ± 0.02 b	0.83 ± 0.00 a
ΨE_0	0.44 ± 0.02 d	0.51 ± 0.00 b,c	0.49 ± 0.01 c	0.56 ± 0.01 a	0.53 ± 0.01 b
φE_0	0.35 ± 0.02 c	0.42 ± 0.00 a,b	0.40 ± 0.01 b	0.43 ± 0.01 a	0.44 ± 0.01 a
δR_0	0.47 ± 0.05 a	0.19 ± 0.01 d	0.29 ± 0.01 c	0.38 ± 0.05 b	0.33 ± 0.01 b,c
φR_0	0.14 ± 0.01 a	0.08 ± 0.01 c	0.11 ± 0.00 b	0.16 ± 0.02 a	0.14 ± 0.00 a
φD_0	0.23 ± 0.01 a	0.17 ± 0.00 b	0.19 ± 0.00 b	0.22 ± 0.02 a	0.17 ± 0.00 b
T_{Fm}	157 ± 16 d	226 ± 9 b,c	165 ± 24 c,d	273 ± 28 a,b	323 ± 14 a
Area	13,850 ± 1,335 c	14,200 ± 408 c	12,750 ± 724 c	24,920 ± 2,381 b	28,980 ± 906 a
Chlorophyll concentration (relative units)					
Chl	7.50 ± 0.58 b	4.80 ± 0.17 c	6.81 ± 0.30 b	13.07 ± 0.88 a	6.75 ± 0.38 b

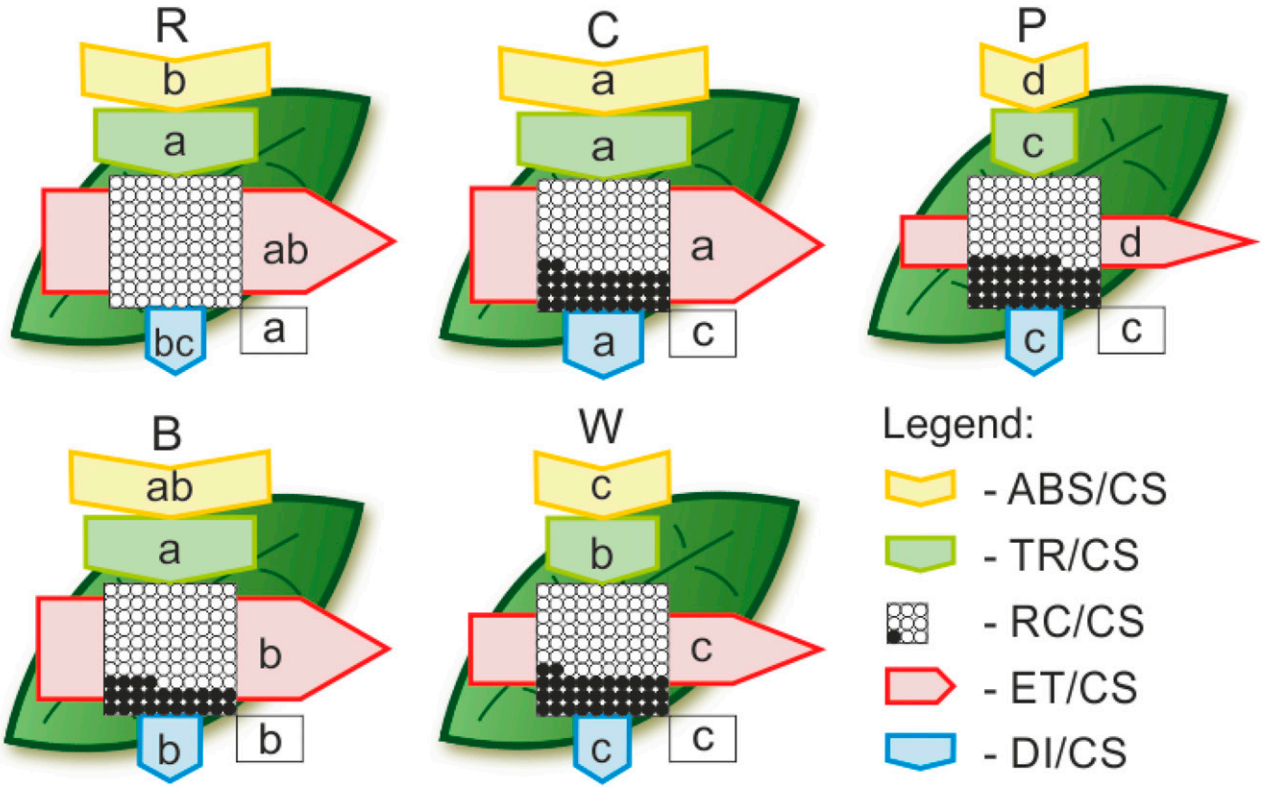

Figure 2. Leaf model showing the phenomenological energy fluxes per the excited CSs of the leaves of the *A. halleri* populations that were studied. Each relative value of the measured parameters is the mean ($n = 60$), and the width of each arrow corresponds to the intensity of the flux. Yellow arrow, ABS/CS, absorption flux per CS approximated; green arrow, TR/CS, trapped energy flux per CS; red arrow, ET/CS, electron transport flux per CS; blue arrow, DI/CS, dissipated energy flux per CS; circles inscribed in squares, RC/CS, percentage of active/inactive RCs. White circles inscribed in squares represent reduced Q_A RCs (active), black circles represent nonreducing Q_A RCs (inactive), and 100% of the active RCs responded with the highest mean value observed in the reference R population. Means followed by the same letter for each parameter are not significantly different from each other using the LSD test ($P \leq 0.05$). Letters are inscribed into arrows, except for RC/CS, where they are placed in a box in the bottom right corner of the square with circles.

and *Sedum alfredii*) and tolerant plants (*Arabidopsis arenosa*) were characterized by a minor although relevant decrease of φP_0 under high HM doses (up to 2,000 μM Zn and 1,000 μM Cd; Cho et al., 2003; Zhou and Qiu, 2005; Küpper et al., 2007; Przedpelska-Wasowicz and Wasowicz, 2013) The nontolerant plants (e.g. *A. thaliana* and *Hordeum vulgare*) were characterized by significant decreases in PSII activity under relatively low doses of Cd (100 μM Cd had a lethal effect; Cho et al., 2003; Kalaji and Loboda, 2007; Maksymiec et al., 2007).

We confirmed the negative impact of HM on energy fluxes per PSII (Fig. 2). Moreover, other authors have shown that Cd strongly interacts with PSII, which causes the deactivation of the RC and, as a result, a decrease in energy trapping and a substantial increase in energy dissipation (Zhou and Qiu, 2005; Kalaji and Loboda, 2007). In hyperaccumulators, the toxic effect of HM on photosynthesis was lower (Zhou and Qiu, 2005) compared with the nontolerant *H. vulgare*, in which 25 μM Cd in the shoots caused the total deactivation of PSII and the loss of energy flux (Kalaji and Loboda, 2007).

Cd caused a flattening of the curves of the chlorophyll fluorescence transient in *A. halleri* M populations (Fig. 1), which may be associated with its toxic effect on the OEC, Q_A pool, and final elements of the electron transport chain, including FNR, which has been reported in a few studies (Kalaji and Loboda, 2007; Kalaji et al., 2014a). The changes in OJIP curves (Fig. 1) showed that HM did not cause the deactivation of PSII in *A. halleri* as was observed in *H. vulgare* (Kalaji and Loboda, 2007).

A. halleri, compared with plants that are not hyperaccumulators, has a much higher tolerance to the toxic effects of HM. Concentrations of HM that almost completely inactivate the PSII of *H. vulgare* or *A. thaliana* (Cho et al., 2003; Kalaji and Loboda, 2007; Kalaji et al., 2014a) only impaired the photosystem negligibly in the case of *A. halleri* (Figs. 1 and 2; Table IV). We found that the M populations that were investigated were characterized by high resistance of their photosynthesis compared with nontolerant plants. Nevertheless, differences in PSII activity within the M populations that were studied were dependent

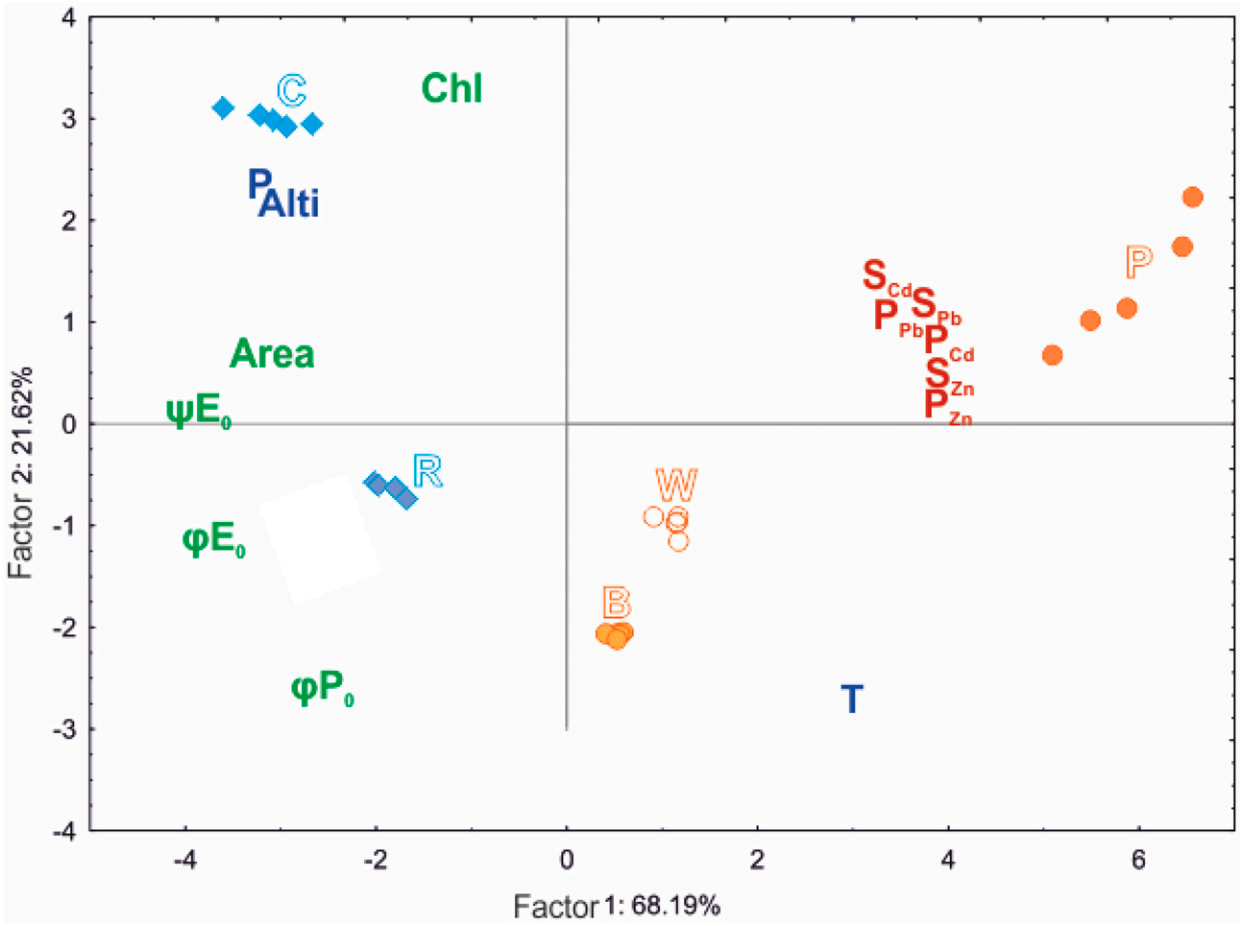

Figure 3. PCA presenting the relationships between the environmental factors, HM concentration in the soil, and accumulation in the shoots and some of the photosynthetic parameters of *A. halleri* populations. The populations are marked with points in different colors and letters. NM *A. halleri* populations (C and R) are grouped in the third and fourth quarters. M populations (B, P, and W) are grouped in the first and second quarters. Parameter designations are as follows: φP_0, maximum quantum yield of the primary PSII photochemistry; ψE_0, probability (at time 0) that a trapped exciton moves an electron into the electron transport chain beyond Q_A; φE_0, quantum yield of electron transport (at time 0); Area, area above the chlorophyll fluorescence curve between F_0 and F_m (reflecting the size of the plastoquinone pool); Chl, chlorophyll content; P, average annual precipitation; Alti, altitude of site; T, annual average temperature; S_{xx}, heavy metal content in the soil; P_{xx}, heavy metal content in the shoots.

on the accumulation of HM in their shoots. The P population, which was characterized by the highest accumulations of Cd, Zn, and Pb, had the lowest yield and activity of PSII.

The physiological status of the plants from population C was very interesting due to the harsh habitat conditions that are connected with growth in an alpine zone. Although the PSII of these plants had the most extensive antennas and the highest electron transport, considerable damage of the RC and high values of dissipation energy were observed (Fig. 2). Furthermore, a high ΔK band was visible on the ΔV_t curves, which was probably connected with OEC damage (Fig. 1B). Population C was characterized by the worst physiological status, which was indicated by the parameter φP_0, which was at the level of the plants from site P (Table IV). Zivcak et al. (2014) demonstrated that plants living in conditions in which there is high light intensity showed an increased content of chlorophyll, number of electron carriers, and higher electron transport between PSII and PSI compared with those living in shade conditions. An increase of chlorophyll content with altitude has been confirmed for many species (Kofidis et al., 2007; Pellissier et al., 2014). Based on the data presented above, it can be hypothesized that the extensive photosynthetic apparatus in mountain plants is an effect of the necessity to accomplish the entire life cycle in only 4 months. Thus, in plants from population C, which grow on an alpine meadow at an altitude of more than 2,000 m AMSL, the extensive antennas and the high electron transport may be connected with the specific environmental conditions. On the other hand, these plants are exposed to extremely high solar radiation and a relatively high dose of UV radiation, which could damage the electron transport chain. Local adaptation by

natural selection is a fundamental process in population differentiation and speciation (Kawecki and Ebert, 2004).

The chlorophyll content in the leaves of the NM and M populations did not differ significantly (Table IV) with the exception of the C (discussed above) and B sites. On the one hand, habitat B is a metalliferous site, while on the other hand, it is on the edge of a forest and under the cover of herbaceous species; therefore, its reduced chlorophyll content could be caused by the shade conditions (Zivcak et al., 2014) and/or the toxic effect of HM on the biosynthesis of the photosynthetic pigment (Myśliwa-Kurdziel and Strzałka, 2002). The lower content of chlorophyll in population B compared with population W, which was caused by Cd treatment, also was observed previously by Meyer et al. (2015) in controlled conditions.

It is tempting to suggest that a wide range of environmental factors, including a wide range of temperatures, precipitation, irradiation, altitude, and HM contamination, affected the diversity within the M and NM populations and caused the appearance of highly specialized ecotypes that differ in their physiological status. It can be hypothesized that differences in photosynthetic efficiency make it improbable to join plants from different habitats into one population, but with a common trait such as the hyperaccumulation of HM, it becomes possible. Stein et al. (2017) confirmed this hypothesis by demonstrating high degrees of differences in the accumulation of HM between each population of *A. halleri* that they investigated.

CONCLUSION

In this study, the relationship between selected photosynthetic efficiency parameters, soil HM concentrations, and environmental factors are presented for *A. halleri* plants in situ, to our knowledge for the first time. The high degree of internal physiological diversity within the populations that inhabit metalliferous and nonmetalliferous sites was presented and proven. In each M population, the Cd and Zn accumulation in the shoots was always higher than in the roots. Regardless of the hypertolerance of *A. halleri* to HM, the toxic effect of Cd, Pb, and Zn on some of the photosynthetic apparatus efficiency parameters such as φP_0, φE_0, and RC/CS was observed. Changes in the PSII parameters, extension of the antennas, and an increased synthesis of the photosynthetic pigment could be physiological responses to the climatic conditions in the alpine zone. Investigations of *A. halleri* that consider plants from different M or NM populations as one group may lead to inconsistent results due to the extremely high physiological diversity within the M and NM populations, which was observed in this study. The ecophysiological studies on *A. halleri* can pave the way to better understanding of the hyperaccumulation phenomenon and the factors that may modify this process.

MATERIALS AND METHODS

Description of the Sites

In this study, selected *Arabidopsis halleri* populations were examined. The selection was based on the variability of environmental conditions in order to present the intraspecies variation in physiological traits (Table I). Natural populations of *A. halleri* were sampled in July 2015 after performing 3 weeks of physiological measurements. The populations were selected due to high environmental differences between them: different altitudes and levels of soil contamination by HM (Table I). The populations were divided into two groups, M and NM (Table I). This division was based on the Cd, Zn, and Pb contents in the soil (Bert et al., 2002; Kabata-Pendias, 2011). On the metalliferous sites, soil contamination was the result of the activity of Zn and Pb smelters. In this study, the abbreviation HM refers to the following elements: Cd, Pb, and Zn.

The M populations that were studied are located in Upper Silesia in southern Poland. They were from 10 to 40 km apart in a straight line. It is noteworthy that Piekary Śląskie (P site) was characterized by the lowest average precipitation (Table I) and the highest HM contamination (Table II) compared with the other habitats that were studied. The second area in terms of contamination was Wełnowiec (W). The soil from site W was characterized by the highest contents of Fe, Mn, and Cu of the tested sites. It also contained relatively high Zn and Pb levels, but these were significantly lower concentrations than the concentrations in soil from site P. Habitat W was characterized by the highest mean annual temperatures and relatively high precipitation. When compared with the other metalliferous sites, the soil that was the least contaminated by HM was in Bukowno (B; Table II). Sites W and B also are known in the literature as PL15 and PL22, respectively (Meyer et al., 2010, 2015).

The NM populations are located in the western Tatra Mountains. Population R (rocky site) inhabits niches in a rock formation (limestone/dolomite) that has a north-facing aspect; as a result, it was not exposed directly to sunlight (Table I). Due to the skeletal character of the soil, it was rich in Mg and Ca, contained trace amounts of phytoavailable HM, and had a slightly alkaline pH (Table II). Population C is located at the summit of Ciemniak Mountain (2,096 m AMSL). The climate is very harsh due to the large fluctuations in daily temperatures, short growing season, the possibility of frost and snowfall all year round, and a large dose of radiation, including UV. The direct genetic relationships of the populations of *A. halleri* that were investigated in this study were confirmed by Pauwels et al. (2005) and Koch and Matschinger (2007). A map with the position and photographs of all investigated sites are included as Supplemental Figures S1 and S2.

Chlorophyll Content and Fluorescence Measurements

Fully developed leaves from the *A. halleri* leaf rosette, which entirely filled the area of the sensor, were selected for the measurements. For each selected plant, measurements were performed for three leaves. The measurements of chlorophyll content and fluorescence were performed on the same leaves. A chlorophyll content meter (CL-01; Hansatech Instruments) was used to measure the chlorophyll content. The chlorophyll content was calculated on the basis of the fluorescence measurement with a 5-mm-diameter probe. Chlorophyll *a* fluorescence measurements were done for the same plants using the Plant Efficiency Analyzer (PocketPEA fluorimeter; Hansatech Instruments). Before being measured, each selected leaf was adapted in the dark for 30 min using leaf clips. After adaptation, a saturating light pulse of 3,500 μmol quanta m^{-2} s^{-1} was applied for 1 s, which closed all of the RCs, and the fluorescence parameters were measured. Both chlorophyll content and fluorescence measurements were performed in situ without destroying the plant material (20 plants per site were examined, giving 60 measurements in total).

Collection and Analysis of the Plant and Soil Samples

At each site, eight individual plants (except site C, where $n = 6$) with a lump of soil to protect the root systems were collected from the 20 plants that were used for the chlorophyll content and fluorescence measurements. At each site, the populations had a large number of specimens, and the sampling that was performed on them did not cause significant injury to their abundance or diversity. In the laboratory, the shoots were separated from the roots and prewashed thoroughly in tap water and then washed using an ultrasound washer filled with deionized water. Each plant sample was dried at 80°C for 72 h to obtain the dry weight. Then, the dry plant material was ground in a mortar, mixed, and acid digested.

For each plant, three samples of the soil from its immediate surroundings were taken and mixed together in order to obtain one composite sample per plant. In the laboratory, the soil samples were air dried and sieved through a 2-mm screen and then used for physicochemical analyses. Soil pH was measured in deionized water (1:2.5, m/v) and 1 M KCl (1:2.5, m/v) with a combination glass/calomel electrode (OSH 10-10; Metron) and a pH/conductivity meter (CPC-505; Elmetron) at room temperature after 24 h of equilibration. The electrical conductivity was determined in a deionized water suspension (soil: solution ratio of 1:2.5, m/v) at room temperature after 24 h of equilibration by using a glass conductivity cell (EC-60; Elmetron) and a pH/conductivity meter (CPC-505; Elmetron). The total metal content was determined after acid digestion of the soil, which had been ground to less than 0.25 mm.

It is generally accepted that the total concentration of metals in soils does not always correlate with their uptake and toxic action in living organisms (Koster et al., 2005). As a consequence, a large number of soil extraction methods have been developed to obtain the bioavailable fraction of metals (for review, see Peijnenburg et al., 2007). Among these, the one-step extraction method of soil with 0.01 M $CaCl_2$, which was proposed by Houba et al. (1996), has proven to be a very useful indication of metal phytoavailability (Pueyo et al., 2004; Meers et al., 2007; Peijnenburg et al., 2007) and has been used by many different laboratories (Hodson et al., 2000; Gucwa-Przepióra et al., 2007; Menzies et al., 2007; Lopareva-Pohu et al., 2011; Vondráčková et al., 2014). For this reason, in this study, the concentration of the phytoavailable forms of the HM in the soil samples was assessed by the extraction of 3 g of dry soil that was sifted to pass through a 2-mm sieve with 30 mL of 0.01 M $CaCl_2$ for 2 h at room temperature.

The plant and soil material was digested in a microwave-assisted wet digestion system (ETHOS 1; Milestone) according to the procedure provided by the manufacturer (concentrated HNO_3 and H_2O_2, 4:1, v/v). The concentration of metals was analyzed in the extracts (soil and $CaCl_2$) and digests (plant and soil) using flame atomic absorption spectrophotometry (iCE 3500 FAAS; Thermo Scientific). Reference plant (Oriental Basma Tobacco Leaves [INCT-OBTL-5]; Institute of Nuclear Chemistry and Technology) and soil material (NCS DC 77302; China National Analysis Center for Iron and Steel) was used for the quality assurance of the analytical data.

Statistical Analyses

The results are shown as means ± SE. The statistical significance of the differences was determined using one-way ANOVA and the posthoc Fisher's LSD test ($P < 0.05$). PCA was used to identify the dominant groups of the factors that determine and describe the habitats. The software used for the statistical analyses was Statistica version 12 (StatSoft). The pipeline models of energy fluxes through the leaf CSs were created using CorelDRAW X6 (Corel).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Overview map of the locations of the investigated sites.

Supplemental Figure S2. Photographs of the investigated sites.

Supplemental Table S1. Correlations between factors examined using PCA.

Supplemental Table S2. Abbreviations and definitions of photosynthesis parameters.

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